Recombinant Human Liver Betaine-homocysteine *S*-Methyltransferase: Identification of Three Cysteine Residues Critical for Zinc Binding†

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Received May 3, 1999; Revised Manuscript Received August 3, 1999

ABSTRACT: Betaine-homocysteine *S*-methyltransferase (BHMT; EC 2.1.1.5) catalyzes the transfer of an *N*-methyl group from betaine to homocysteine to produce dimethylglycine and methionine, respectively. The enzyme is found in the pathway of choline oxidation and is abundantly expressed in liver and kidney. We have recently shown that human BHMT is a zinc metalloenzyme [Millian, N. S., and Garrow, T. A. (1998) *Arch. Biochem. Biophys. 356*, 93−98]. To facilitate the rapid purification of human BHMT for further physical and mechanistic studies, including characterizing its metal binding properties, we have overexpressed the enzyme in *E. coli* as a fusion construct which facilitated its subsequent purification by a self-cleavable affinity tag system (IMPACT T7). Using this expression and purification system in conjunction with site-directed mutagenesis, we have identified Cys217, Cys299, and Cys300 as zinc ligands. Mutating any of these Cys residues to Ala results in the complete loss of activity and a significant reduction in the ability of the protein to bind zinc. Comparing the regions of BHMT amino acid sequence surrounding these Cys residues with similar amino acid sequences retrievable from protein databases, we have identified the following motif: G[ILV]NCX(20,100)[ALV]X(2)[ILV]GGCCX(3)PX(2)I, which we propose to be a signature for a family of zinc-dependent methyltransferases that utilize thiols or selenols as methyl acceptors. Some of the members of this family include the vitamin B_{12}-dependent methionine synthases, *E. coli* *S*-methylmethionine-*S*-homocysteine methyltransferase, and *A. bisulcatus* *S*-methylmethionine-selenocysteine methyltransferase.

Betaine-homocysteine *S*-methyltransferase (BHMT;[1] EC 2.1.1.5) is a cytosolic (*1*) enzyme found in the pathway of choline oxidation. This enzyme catalyzes a methyl transfer from betaine to homocysteine to form dimethylglycine and methionine, respectively. Initial rate studies with the rat liver enzyme indicated that it uses an ordered Bi-Bi kinetic mechanism whereby homocysteine is the first substrate to bind and methionine is the last product off (*2*). The enzyme is a hexamer of identical subunits (*3*) and is expressed at very high levels in the liver and kidney cortex (*4*).

A cDNA encoding human liver BHMT was cloned and sequenced by our lab several years ago (*5*), and its deduced amino acid sequence was shown to have limited homology to the N-terminal region of vitamin B_{12}-dependent methionine synthases (B_{12}-MS, EC 2.1.1.13), another enzyme that methylates homocysteine. Following this report, Goulding et al. (*6*) showed that *E. coli* B_{12}-MS was a zinc (Zn) metalloenzyme and that mutating either Cys310 or Cys311 to Ala, a region previously shown to be conserved in BHMT (*5*), dramatically reduced Zn binding and catalytic activity. Shortly thereafter, we developed a purification protocol for recombinant human liver BHMT expressed in *E. coli* and showed that it too was a Zn metalloenzyme that could be depleted of the metal using the thiol-specific reagent methylmethane thiosulfonate (*7*). More recently, it has been shown that mutating Cys247 to Ala in *E. coli* B_{12}-MS also dramatically reduced Zn binding and catalytic function (*8*). Furthermore, extended X-ray fine structure analysis of the wild-type protein confirmed that the Zn in B_{12}-MS was coordinated by three thiolates, consistent with the Cys247Ala, Cys310Ala, and Cys311Ala mutation studies. Since both BHMT and the N-terminal domain of *E. coli* B_{12}-MS share limited homology, catalyze the methylation of homocysteine, and are Zn-dependent enzymes, it seems likely that they also share similar mechanisms to bind and activate homocysteine for S-methylation.

In this report we present a new one-step affinity purification procedure for recombinant human liver BHMT and describe a new in vivo microbiological assay for its activity. Using these new techniques in combination with the standard radioassay for BHMT activity and site-directed mutagenesis, we have determined that Cys217, Cys299, and Cys300 of BHMT are required for Zn binding and catalytic function.

† This research was supported in part by the University of Illinois' Agricultural Experiment Station (60-0305), and by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (DK52501) and the National Heart, Blood, and Lung Institute (HL58955).

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[1] Abbreviations: Bet, betaine; BHMT, betaine-homocysteine *S*-methyltransferase; BME, β-mercaptoethanol; B_{12}-MS, vitamin B_{12}-dependent methionine synthase; Dmg, dimethylglycine; EDTA, ethylenediaminetetraacetic acid; Gly, glycine; Hcy, homocysteine; ICP, inductively coupled plasma emission spectrometry; IPTG, isopropyl β-D-thiogalactopyranoside; Met, methionine; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Ser, serine; H_4folate, tetrahydrofolate; Tris, tris(hydroxymethyl)aminomethane; Zn, zinc.

10.1021/bi991003v CCC: $18.00
Published on Web 09/24/1999

Table 1: Sequences of PCR Primers Used for Cloning and Mutagenesis[a]

primer	sequence
FP-hBHMT	CGTATAATGTGTGGAATTGTGAGCGG
RP-hBHMT	CTGTGATTTGAATTTTTGTTTTTCAAAGAGCTCTTTCAGCTGCTGC
FP-N216A	GCAGGAGCATCCATCATTGGTGTGgcCTGtCACTTTGACCCC
RP-N216A	GGGGTCAAAGTGaCAGgcCACACCAATGATGGATGCTCCTGC
FP-N216C	GCAGGAGCATCCATCATTGGTGTGtgCTGtCACTTTGACCCCACC
RP-N216C	GGTGGGGTCAAAGTGaCAGcaCACACCAATGATGGATGCTCCTGC
FP-C217AS	GCAGGAGCATCCATCATTGGTGTtAACkCCCACTTTGACCCC
RP-C217AS	GGGGTCAAAGTGGGmGTTaACACCAATGATGGATGCTCCTGC
FP-C217H	GCAGGAGCATCCATCATTGGTGTtAACcaCCACTTTGACCCC
RP-C217H	GGGGTCAAAGTGGtgGTTaACACCAATGATGGATGCTCCTGC
FP-D220A	GGTGTGAACTGtCACTTTGcaCCCACCATTAGTTTAAAAACAGTGAAGC
RP-D220A	GGTGGGtgCAAAGTGaCAGTTCACACCAATGATGGATGCTCCTGC
FP-C299AS	GGGGTCAGGTACATTGGCGGckccTGTGGATTTGAGCCCTACCACATCAGGGC
RP-C299AS	GGGCTCAAATCCACAggmgCCGCCAATGTACCTGACCCCCAGGTTGTAGGCC
FP-C300AS	GGGGTCAGGTACATTGGCGGGTGCkccGGcTTTGAGCCCTACCACATCAGGGC
RP-C300AS	GGGCTCAAAgCCggmGCACCCGCCAATGTACCTGACCCCCAGGTTGTAGGCC
FP-E303A	GGCGGGTGCTGTGGATTTGcGCCCTACCACATCAGGGC
RP-E303A	GATGTGGTAGGGCgCAAATCCACAGCACCCGCCAATGTACC
FP-H306A	CGGGTGCTGTGGATTTGAGCCgTACgcCATCAGGGCAATTGCAGAGGAGCTGGCC
RP-H306A	GGCCAGCTCCTCTGCAATTGCCCTGATGgcGTAcGGCTCAAATCCACAGCACCCG

[a] All primers were synthesized by Integrated DNA Technologies and are listed 5′to 3′. Lower case letters denote a mutation, and m and k equal a or c and g or t, respectively. FP identifies primers complementary to the coding strand, and those complementary to the noncoding strand are identified as RP. Using *Pfu* polymerase under standard PCR conditions, the cDNA of BHMT was amplified from pTrc99A-hBHMT with primers FP-hBHMT and RP-hBHMT. Site-directed mutagenesis was conducted using the QuikChange kit (Stratagene) with either pTrc99A-hBHMT or pTBY4-hBHMT as the template.

These Cys residues of BHMT align to Cys247, Cys310, and Cys311 of *E. coli* B_{12}-MS, previously shown to be the Zn ligands in that protein. By comparing the known amino acid sequences of BHMT and B_{12}-MS near the Cys residues required for Zn binding, we show that the motif G[ILV]-NCX(20,100)[ALV]X(2)[ILV]GGCCX(3)PX(2)I can be used to identify other Zn-dependent methyltransferases that utilize thiols or selenols as the methyl acceptors.

EXPERIMENTAL PROCEDURES

Materials. Hybond-N nylon transfer membranes were purchased from Amersham Corp. Ampicillin, isopropyl β-D-thiogalactopyranoside (IPTG), ethylenediaminetetraacetic acid (EDTA), and β-mercaptoethanol (BME) were obtained from Sigma. Radiolabeled [^{14}C]dimethylacetothetin was synthesized as previously described (*5*), and radiolabeled [^{14}C]betaine was a gift from Dr. James Finkelstein (VA Medical Center, Washington, DC). All other reagents were of the highest analytical or molecular grade available from commercial vendors.

Bacterial strain J5-3 (CGSC #6850) was obtained from the *E. coli* Genetic Stock Center at Yale University (New Haven, CT). All primers were synthesized by Integrated DNA Technologies and are shown in Table 1. DNA was sequenced at the University of Illinois' Biotechnology Center (Urbana, IL). The IMPACT (intein-mediated purification with an affinity chitin-binding tag) T7 system and the chitin beads were purchased from New England Biolabs.

Preparation of the BHMT-Intein/Chitin-Binding Fusion Construct (pTYB4-hBHMT). The cDNA sequence of human liver BHMT (accession number U50529) and its cloning into the *E. coli* expression vector pTrc99A (Pharmacia Biotech) to form pTrc99A-hBHMT have been previously described (*5*, *7*). For this construct, a *Nco*I restriction site was engineered at the first ATG of the cDNA. Standard PCR conditions were employed to amplify BHMT from pTrc99A-hBHMT using *Pfu* polymerase (Stratagene) and two oligonucleotide primers: FP-hBHMT and RP-hBHMT. FP-hBHMT was complementary to the 5′-flanking region of the amino-terminal coding strand, whereas RP-hBHMT was complementary to the carboxy-terminal noncoding strand and started from the penultimate residue from the stop codon. Synthesis of the desired PCR product was verified by agarose electrophoresis and then purified using a PCR Quick Spin Kit (Qiagen). The PCR product was digested with *Nco*I, and pTYB4 was digested with *Nco*I and *Sma*I. Following gel purification of the DNA fragments, T_4 DNA ligase (Gibco) was used to ligate the PCR product into pTYB4 (New England Biolabs). The ligase reaction product was then ethanol-precipitated, resuspended in water, and transformed into *E. coli* strain XL1-Blue (Stratagene). The resulting expression construct was verified by DNA sequencing and named pTYB4-hBHMT.

Mutagenesis of BHMT. Site-directed mutagenesis of BHMT was performed using a QuikChange kit (Stratagene) with either pTYB4-hBHMT or pTrc99A-hBHMT as the template. The PCR primers used to generate each mutation are listed in Table 1, and all mutations were verified by DNA sequencing.

Expression of Wild-Type and Mutant BHMTs Using the pTrc99A-hBHMT Constructs. Fifty milliliters of 2×YT media containing 250 μM Zn chloride and 0.10 mg/mL ampicillin was inoculated with an overnight culture of JM105 (New England Biolabs) transformed with either wild-type or mutant pTrc99A-hBHMT constructs. After growing to A_{600} = 0.6 at 37 °C, the culture was induced with 3 mM IPTG and incubated for an additional 3 h. Following the induction period, the cells were collected by centrifugation at 5000*g*

for 30 min (4 °C) and resuspended in 5 mL of buffer containing 100 mM Tris (pH 8.2) and 10 mM BME. The resuspended cells were then incubated with egg white lysozyme (Amersco) at a final concentration of 0.8 mg/mL for 30 min (23 °C). The cells were then completely lysed by sonication. Cell debris was removed by centrifugation at 15000*g* for 30 min (4 °C), and the clarified lysate was analyzed for BHMT by activity and Western analyses.

In Vivo Assay for BHMT Activity. E. coli strain J5-3, a methionine auxotroph due to a lack of methylenene H_4folate reductase (EC 1.1.1.68) activity, was transformed with wild-type or mutant pTrc99A-hBHMT constructs and plated on 2×YT media containing 0.10 mg/mL ampicillin. Single colonies were then streaked on Vogel−Bonner minimal media (*9*) plates that were supplemented with 0.10 mg/mL ampicillin, 50 mg/L L-proline, 0.001% thiamin, 500 mM sodium chloride, 3 mM IPTG, and either 10 mM dimethylacetothetin or 10 mM betaine. Plates were incubated at 37 °C for 2−4 days and then scored for growth.

Expression and Purification of Wild-Type and Mutant BHMTs Expressed Using the pTYB4-hBHMT Constructs. Two liters of 2×YT media containing 250 μM Zn chloride and 0.10 mg/mL ampicillin was inoculated with an overnight culture of BL21(DE3) (Novagen) transformed with wild-type or mutant pTYB4-hBHMT plasmids. After growing to A_{600} = 1.0−1.5 at 37 °C, the culture was rapidly cooled to 16 °C, induced with 0.3 mM IPTG, and incubated for an additional 16 h at 16 °C. Following the induction period, the cells were collected by centrifugation at 5000*g* for 10 min (4 °C) and resuspended in ice-cold buffer containing 20 mM Tris (pH 8.0), 500 mM sodium chloride, 0.1 mM EDTA, and 0.1% Triton X-100. The cells were then lysed by sonication, and the lysate was clarified by centrifugation at 15000*g* for 30 min (4 °C). The enzyme was then purified at 4 °C using a chitin affinity column as described below.

The clarified lysate was applied (0.5 mL/min) to a chitin affinity column (10 mL) that had been equilibrated with buffer containing 20 mM Tris (pH 8.0), 500 mM sodium chloride, 0.1 mM EDTA, and 0.1% Triton X-100. The column was then washed with 300 mL of the same buffer (1 mL/min) followed by 100 mL of buffer containing 20 mM Tris (pH 8.0), 50 mM sodium chloride, and 0.1 mM EDTA. The column was then rapidly washed with 30 mL of buffer containing 20 mM Tris (pH 8.0), 50 mM sodium chloride, 0.1 mM EDTA, and 30 mM BME (5 mL/min). The column was then capped and allowed to stand for 16 h. Then, BHMT was eluted from the column using buffer containing 20 mM Tris (pH 8.0), 50 mM sodium chloride, 0.1 mM EDTA, and 30 mM BME. Fractions were analyzed by SDS−PAGE in combination with Coomassie blue staining. Fractions which exhibited a single band at 45 kDa were pooled. The protein concentration of the purified enzyme was then determined by a Coomassie dye-binding assay (Bio-Rad Laboratories) using bovine serum albumin as standard.

In Vitro Assay for BHMT Activity. The standard radioassay for BHMT activity has been previously described (*5*). The final concentrations of assay components were 5 mM DL-homocysteine, 2 mM betaine (0.1 μCi), 5 mM BME, and 50 mM Tris (pH 7.5). The reaction was initiated with the addition of sample to give a total volume of 0.5 mL, and the reactants were incubated at 37 °C for 0.5−2 h. Some assays were supplemented with zinc chloride (500 μM), and in the case when more sensitive measurements were required, betaine was replaced with 2 mM dimethylacetothetin (0.082 μCi) and incubations were extended to 5 h. For each assay, blanks were prepared from the same buffer conditions as the sample to be measured.

SDS−PAGE and Western Analysis Conditions. SDS−PAGE analysis was conducted using a using a 5% stacking and a 12% separation gel in combination with a Tris−glycine discontinuous buffer system. Following electrophoresis, gels were stained with Coomassie blue. For Western analysis, protein from the gel was blotted onto nitrocellulose using a Tris−glycine−methanol transfer buffer and a semi-dry blotting apparatus (Bio-Rad Laboratories). The blot was probed for BHMT protein using rabbit polyclonal antibodies prepared against highly purified recombinant hBHMT (*10*). The antigen−antibody interaction was detected using peroxidase-coupled anti-rabbit IgG (goat) and tetramethylbenzidine as the peroxidase substrate (Vector Laboratories).

Metal Content Determination by Inductively Coupled Plasma Emission Spectrometry (ICP). PD-10 columns (Pharmacia BioTech) were equilibrated with buffer containing 10 mM Tris (pH 7.2) and 10 mM BME that had been scrubbed of metals by passage over Chelex 100 resin (Bio-Rad Laboratories). Protein samples (1.5 mL) that had been previously affinity-purified were loaded into these PD-10 columns and eluted with the addition of metal-free buffer containing 10 mM Tris (pH 7.2) and 10 mM BME. One-milliliter fractions were collected, and for each column run, the protein eluted in fractions 4, 5, and 6 as determined by the Coomassie dye-binding assay and SDS−PAGE analysis. Fractions 4 and 5 were pooled and submitted for Zn analysis by ICP. The blank consisted of an aliquot of buffer [100 mM Tris (pH 8.2) and 10 mM BME] that was treated in the same manner as the protein samples. The standards were prepared in buffer containing 10 mM Tris (pH 7.2) and 10 mM BME. ICP measurements were conducted at the Micro Analysis Facility at the University of Illinois (Urbana, IL).

Blast Searches of GenBank. GenBank was searched using a Pattern Hit Initiated Blast (*11*) with the amino acid sequence of human BHMT as the subject sequence and GGCC as the pattern. In cases where multiple sequences were retrieved encoding the same enzyme but from different species, as in the case of B_{12}-MS, only one was chosen for comparison to limit redundancy. Modifications of the Blast alignments were made by hand.

RESULTS

Preparation of the BHMT-Intein/Chitin-Binding Fusion Construct pTYB4-hBHMT. The open reading frame of human BHMT was PCR-amplified and ligated into pTYB4 to form the BHMT-intein/chitin binding fusion construct (pTYB4-hBHMT). This plasmid was transformed into *E. coli* BL21(DE3) for the bacteriophage T7 promoter-driven expression of the fusion protein. In this construct, the C-terminus of BHMT is fused to the N-terminus of *S. cerevisiae's* intein gene, which in turn is fused to *B. circulans'* chitin-binding domain, the latter of which is required for affinity purification. Upon BME-induced autocleavage of the BHMT-intein fusion, BHMT is left with an additional C-terminal glycine residue (residue 407) which is not found on the native 406 residue protein. Mutant forms

Table 2: BHMT Activities and Zn Content of Wild-Type and Mutant Proteins

enzyme	in vitro[a]	[Zn]:[BHMT][b]	in vivo[c]
wild type	37	1.00	+
N216A	15	nd	+
N216C	4	nd	+
C217A	0	0.05	−
C217S	0	0.21	−
C217H	0	0.66	−
D220A	27	nd	+
C299A	0	0.22	−
C299S	0	0.18	−
C300A	0	0.08	−
C300S	0	nd	−
E303A	23	nd	+
H306A	21	0.76	+

[a] BHMT was expressed using the pTrc99A-based system. Conditions for the standard betaine-dependent radioassay are described in detail under Experimental Procedures. Each assay contained 2 mM betaine (0.05 mCi), 5 mM DL-homocysteine, and 4−8 mg of *E. coli* lysate. Activities in lysates are listed as nanomoles per hour per milligram. [b] Wild-type BHMT and some mutant enzymes were expressed and purified using the pTYB4-based system. Proteins were then analyzed for Zn content as described under Experimental Procedures. nd indicates "not determined". These proteins (~0.9 mg of protein) were also tested for activity using the betaine-dependent radioassay. All Cys mutants were inactive. The only mutant tested that had activity was H306A, which had a specific activity that was 55% of the wild-type enzyme. [c] Conditions for the microbiological in vivo assay are described under Experimental Procedures. The assay selects for BHMT-dependent methionine production using the *E. coli* J5-3 methionine auxotroph harboring clones expressing BHMT using the pTrc99A-based system. A plus sign indicates robust bacterial growth, whereas a minus sign indicates no bacterial growth (see also Figure 2B).

of the enzyme were prepared by site-directed mutagenesis and overexpressed in the same manner.

Expression of Wild-Type and Mutant BHMTs Using the pTrc99A-hBHMT Constructs and in Vitro Activity Analysis. As an initial test for activity, small cultures of JM105 transformants were induced with IPTG to express the wild-type or mutant BHMT proteins based on the pTrc99A-hBHMT constructs. Western analysis of the crude lysates verified that full-length enzymes were expressed, and analysis for BHMT activity using betaine as the methyl donor substrate revealed that Cys217Ala, Cys217Ser, Cys217His, Cys299Ala, Cys299Ser, Cys300Ala, and Cys300Ser were inactive. All other mutants had activities similar to the wild-type enzyme (Table 2).

Microbiological Screen for BHMT Activity. An in vivo assay for BHMT activity was developed based on complementation of the methionine auxotrophy of *E. coli* strain J5-3 (see Figure 2A). Transformed with wild-type and mutant pTrc99A-hBHMT constructs, single colonies were streaked out on supplemented Vogel−Bonner plates devoid of methionine and incubated at 37 °C. The plates were then scored for growth. Examination of growth at a wide range of betaine and dimethylacetothetin concentrations revealed that the maximal growth rate was obtained using 10 mM dimethylacetothetin. In addition, plates supplemented with dimethylacetothetin could be scored after 2 days, whereas those supplemented with betaine required 4 days, consistent with the enzyme displaying greater catalytic efficiency toward thetin analogues (*5*). Wild-type BHMT, Asn216Ala, Asn216Cys, Asp220Ala, Glu303Ala, and His306Ala complemented the methionine auxotrophy and displayed no discernible difference in growth rate, whereas all the other mutant proteins could not support growth (Table 2 and Figure 2B).

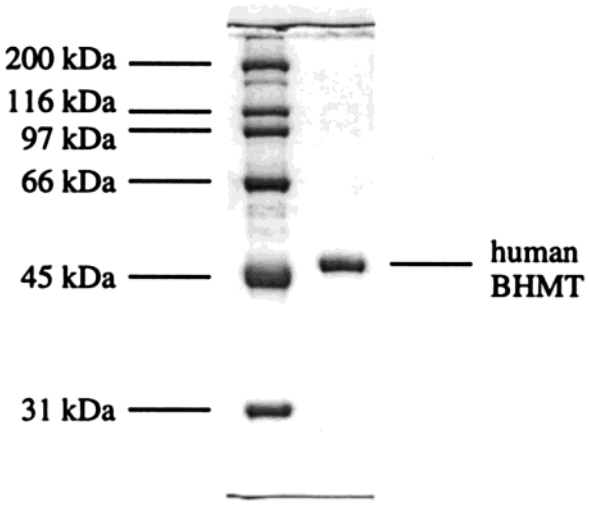

FIGURE 1: SDS−PAGE analysis of purified BHMT. Left lane: bovine carbonic anhydrase, 31 kDa; hen egg white ovalbumin, 45 kDa; bovine serum albumin, 66 kDa; rabbit muscle phosphorylase B, 97 kDa; *E. coli* β-galactosidase, 116 kDa; rabbit skeletal muscle myosin, 200 kDa. Right lane: wild-type recombinant human liver BHMT (2.4 μg) purified using the pTBY4-based system.

Expression and Purification of Wild-Type and Mutant BHMTs Using the pTYB4-hBHMT Constructs and Metal Content Determination by ICP. BL21(DE3) cells were transformed with pTYB4-hBHMT constructs and grown in 2×YT media supplemented with 250 μM zinc chloride. Upon IPTG induction, the cells overproduced a soluble BHMT-intein/chitin-binding fusion protein. Time course and temperature dependence studies revealed that induction for 16 h at 16 °C resulted in maximal expression. Following the one-step affinity purification protocol, 10−15 mg of enzyme could be collected from 1 L of culture. The following proteins were affinity-purified: wild-type, Cys217Ala, Cys217Ser, Cys217His, Cys299Ala, Cys299Ser, Cys300Ala, and His306Ala. SDS−PAGE analysis of the wild-type enzyme can be seen in Figure 1. BHMT migrated at about 45 kDa, consistent with its deduced amino acid sequence and our previous results obtained for the purified enzyme expressed in *E. coli* using the pTrc99A-based system (*7*). All mutant proteins were identical in size to the wild-type enzyme as judged by SDS−PAGE and Western analyses (not shown). The affinity-purified wild-type enzyme also had a specific activity that was indistinguishable from the Zn-replete recombinant human liver enzyme previously purified (*7*), both of which were similar to the native enzymes purified from rat (*3*), pig (*5*), and human (*12*) livers. The Cys217, Cys299, and Cys300 mutants were all inactive using the betaine-dependent radioassay, whereas the His306Ala mutant had a specific activity that was 55% that of the wild-type enzyme. The purified proteins were also analyzed for Zn content by ICP. The wild-type enzyme had a Zn-to-monomer ratio of 1.0, which is in good agreement with our previously reported value of 0.9 (*7*). However, with the exception of Cys217His and His306Ala, each of the mutant proteins tested contained significantly lower levels of Zn per protein monomer (Table 2).

In Vitro Assay of Cys217Ser, Cys217His, and Cys299Ser BHMT Proteins Using Dimethylacetothetin as Methyl Donor.

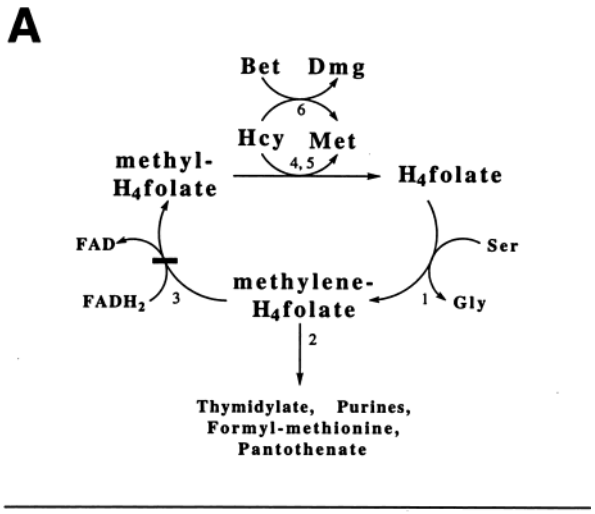

FIGURE 2: Panel A: Bypassing folate-dependent methionine biosynthesis in *E. coli* strain J5-3 with human BHMT. Reactions: 1, serine hydroxymethyltransferase; 2, miscellaneous reactions that utilize one-carbon units from the folate pool; 3, methylene H_4folate reductase; 4, methionine synthase (vitamin B_{12}-independent); 5, methionine synthase (vitamin B_{12}-dependent); and 6, human BHMT. The black bar represents the metabolic block at reaction 3 in *E. coli* strain J5-3. Panel B: The in vivo microbiological assay of wild-type and mutant BHMT proteins by complementing the methionine auxotrophy of *E. coli* strain J5-3. The details of the assay are described under Experimental Procedures.

Assays using dimethylacetothetin in place of betaine were conducted with purified BHMT proteins Cys217Ser, Cys217His, and Cys299Ser to determine if the mutants retained any residual BHMT activity below the detection limits of the betaine-dependent in vitro assay. In addition, samples (~0.9 mg of protein) were incubated at 37 °C for an extended period of time (5 h). The reaction tubes also contained Zn at a final concentration of 500 μM. Compared to the wild-type enzyme, Cys217Ser and Cys299Ser were found to retain 0.30 and 0.21% activity, respectively. Protein Cys217His did not have any detectable activity.

Blast Searches of GenBank. Some targets for site-directed mutagenesis were chosen using protein alignment information. GenBank was searched by a Pattern Hit Initiated Blast using the amino acid sequence of human BHMT as the subject sequence and GGCC as the pattern. The search yielded 33 protein similarities. Six sequences besides BHMT were chosen, and the alignments were adjusted by hand to reveal four conserved regions (A, B, C, and D) that were shared by all seven enzymes. A fifth region (C2) was shared with all of the enzymes except BHMT (Figure 3). Regions C and D are of particular interest because they contain the putative Zn ligands of BHMT.

DISCUSSION

We previously showed that BHMT shared limited homology to B_{12}-MS proteins (*5*) and more recently that it was a Zn metalloenzyme (*7*). Furthermore, BHMT could be depleted of Zn using the thiol-specific reagent methylmethane thiosulfonate, suggesting that Cys residues were involved in Zn binding. *E. coli* B_{12}-MS, another enzyme that methylates homocysteine, has also been shown to be Zn-dependent (*6*). In addition, it has been shown that three Cys residues provide thiolates for Zn binding in B_{12}-MS (*6*, *8*). The goals of this study were to determine what residues are required for Zn binding in BHMT, and to find a residue(s) that has (have) a general acid or base role in BHMT catalysis. Although we previously purified the recombinant human liver enzyme to homogeneity, the method employed required multiple chromatographic steps and several days to complete (*7*). To more rapidly evaluate the effects of site-directed mutagenesis on metal binding and protein structure and function, we developed a high-expression−rapid purification procedure for BHMT and a new in vivo assay for enzyme activity designed to facilitate mutant screening.

The new system employed to overexpress and purify human BHMT was IMPACT T7 (New England Biolabs). In brief, the open reading frame of the BHMT cDNA was ligated into pTYB4 to create pTYB4-hBHMT. Transformation of pTYB4-hBHMT into BL21(DE3) resulted in a high level of expression of a soluble BHMT fusion protein following IPTG induction. The fusion protein was then separated from other lysate proteins by binding onto a chitin affinity column. Then, BHMT was specifically eluted from the column by the BME-induced intein-mediated self-cleavage, which releases BHMT from its fusion partner.

To probe BHMT for residues essential for catalytic function, it was desirable to have a sensitive assay for enzyme activity. The sensitivity of the standard radioassay for detecting BHMT activity is limited by the very low V_{max} of the enzyme (~2 μmol h^{-1} mg^{-1}); thus, high specific activity [^{14}C-*methyl*]betaine and prolonged incubation periods are required for the detection of low levels of activity. The sensitivity of the in vitro radioassay can be improved by using radiolabeled dimethylacetothetin as the methyl donor substrate. Dimethylacetothetin is the direct sulfonium analogue of betaine, and BHMT displays an approximately 50-fold greater V_{max} using dimethylacetothetin when compared to betaine (*5*). Although the higher catalytic efficiency of BHMT toward dimethylacetothetin improves the ability to detect activity, the enzyme still turns over relatively slow. Therefore, as an alternative to the in vitro measurements of BHMT activity and with the hope of increasing the sensitivity of detecting residual activity, an in vivo microbiological screen for enzyme activity was developed. The microbiological assay was based on complementing the methionine auxotrophy of *E. coli* strain J5-3 with BHMT-dependent methionine production. The methionine auxotrophy of J5-3 is caused by a lack of methylene H_4folate reductase activity, which is required to provide the methyl H_4folate needed by *E. coli's* B_{12}-dependent and B_{12}-independent methionine synthases (Figure 2A). We found that when J5-3 was transformed with a plasmid that allowed the IPTG-inducible expression of BHMT (pTrc99A-hBHMT), the enzyme-catalyzed reaction could complement the methionine require-

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hBHMT             MPPVGGKKAKKGILERLNAGEIVIGDGGFVFALEKRGYVKAGPWTPEAAVEH--------------   52
ecMS              MSSKVEQLRAQLN-ER-----ILVLDGGMGTMIQSYRLNEADFRGERFADWPCDLKGNNDLLVLSK   58
YLL062c           MKRIPIKE---LIVEHPGK--VLILDGGQGTELENRGININSPVWSAAPFTSESFWEPSSQE----   57
YPL273w           MARLPLKQ---FLADNPKK--VLVLDGGQGTELENRGIKVANPVWSTIPFISESFWSDESSAN---   58
bsYitJ            M----------GLLEDLQRQ-VLIGDGAMGTLLYSYGIDRCFEELNISK-----------------   38
abSmtA            MSSPLITDFLHQAGR------AAVIAGGLGTELQRHGADLNDPLWSAKCLLSC-------------   47
ecYagD            MSQNNPLR---ALLDKQD---ILLLDGAMATELEARGCNLADSLWSAKVLVEN-------------   47
                                      *****G************
                                           Region A


PEAVRQLHREFLRAGSNVMQTFTFYASEDKLENR- 86 -KAGASIIGVNCHFDPTISLKTVKLMKEGLEAAQLKAHLMSQPLA  250
PEVIAAIHNAYFEAGADIIETNTFNSTTIAMADY- 94 -HAEALTFGLNCALGPDELRQYVQELSRIAECYVTAHPNAGLPNA  280
RKVVEEMYRDFMIAGANILMTITYQANFQSISEN- 91 -NKNLLLMGVNCVSFNQSALILKMLHEHLPGMPLLVYPNSGEIYN  271
RKIVKEMFNDFLNAGAEILMTTTYQTSYKSVSEN- 92 -NPNFSFLGINCVSFNQSPDILESLHQALPNMALLAYPNSGEVYD  272
PEEIQRIHKAYVEAGANIIQTNTYGANYIKLSRH- 72 -DLGADIVGINCRLGPYHMIEALSEVPIFDDVFLSVYPNSSLPSL  233
PHLIRQVHLDYLENGADIIITASYQATIQGFKAK- 81 -CDKVVAVGINCTPPRFIHDLILLLKKVTAK-PIVIYPNSGETYD  277
PELIREVHLDYYRAGAQCAITASYQATPAGFAAR- 81 -YPQVVALGINCIALENTTAALQHLHGLTVL-PLVVYPNSGEHYD  261
**************G*****T*************           **G*NC*                   ****PN***
              Region B                       Region C                  Region C2


WDIQKYAREAYNLGVRYIGGCCGFEPYHIRAIAEELAPER...318
TMAKQIREWAQAGFLNIVGGCCGTTPQHIAAMSRAVEGLA...329
DDWETTVKKFVDNGARIIGGCCRTSPKDIAEIASAVDKYS...324
NSWDTVVKQYISSGARIIGGCCRTSPKDIQEISAAVKKYT...325
TYFQNSASEFRKQGARIIGGCCGTTPNHIRAMAEAVGGLA...284
EDFVSYVDKWCESGASLVGGCCRTTPDTIRGIYKILSSGQ...331
AQLADYLPQWQAAGARLIGGCCRTTPADIAALKARS.......310
             *****GGCC***P**I***
                  Region D
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FIGURE 3: Regional alignment of known and potential Zn-dependent methyltransferases that use thiol and/or selenol methyl acceptors. The amino acid sequences are human BHMT (hBHMT: g1522683), *E. coli* B_{12}-MS (ecMS: g2144394), two yeast open reading frames (YLL062c: g1077317; YPL273w: g1370563), and one *B. subtilis* open reading frame (bsYitJ: g2145402) of unknown function, *A. bisulcatus* *S*-methylmethionine-selenocysteine methyltransferase (abSmtA: g40068), and *E. coli* *S*-methylmethionine-homocysteine methyltransferase (ecYagD: g2495491). Numbers indicate the position of the last residue in a given region. Sequences were identified using region D of hBHMT for a Blast search of GenBank. The three Cys residues in regions C and D are required for Zn binding in human BHMT and *E. coli* B_{12}-MS. Asterisks below the alignment indicate conserved regions, and complete residue conservation is denoted by the appropriate amino acid single-letter code. Region C2 is underlined to indicate that this region is found in all of the sequences except hBHMT.

ment of J5-3 when grown in media containing 10 mM betaine or 10 mM dimethylacetothetin. Cells grown with dimethylacetothetin required 2 days to detect growth, whereas those grown with betaine required 4 days. This assay was convenient for the qualitative determination of BHMT activity, and we used it in combination with the in vitro radioassays to confirm which mutations resulted in inactive enzymes.

After the development of a rapid purification system for BHMT, and the development of the in vivo assay for enzyme activity to complement the standard radioassays readily available, we then began to evaluate the effects of mutating specific residues in BHMT on its methyltransferase activity and Zn binding ability. The pTrc99A-based constructs were used for the in vivo microbiological assay because that expression vector uses a trp-lac fusion promoter which can be recognized by J5-3 cells. The IMPACT T7-based overexpression and purification system for BHMT, which uses a T7 promoter and thus requires genetically modified cells expressing T7 RNA polymerase, was then used to rapidly isolate any protein in sufficient quantities to determine metal content. The purified proteins were then also available to test for any subtle changes in catalytic efficiency by the standard radioassays, since small changes in catalytic activity would be missed by the microbiological assay.

Residues targeted for mutagenesis were identified using protein alignment software. Since the deduced amino acid sequences of the four known BHMT sequences (human, rat, mouse, and pig) are highly conserved (>95%), no insight could be gained as to which residues might have a role in the structure and function of the enzyme. A similar problem existed when aligning the known amino acid sequences for B_{12}-MS. However, as noted above, when we previously compared the amino acid sequences of human BHMT and *E. coli* B_{12}-MS, we identified the conserved sequence of GGCCG (*5*). The Cys residues in this sequence correspond to 299 and 300 in human BHMT, and 310 and 311 in *E. coli* B_{12}-MS. Subsequently, Goulding et al. (*6*) identified Cys310 and Cys 311 as ligands for Zn in B_{12}-MS, and later it was determined that Cys247 was a third thiolate ligand (*8*). Cys247 of B_{12}-MS did not readily align with any Cys residue in BHMT when analyzed by a number of computer-based alignment programs. However, when BHMT was aligned by hand with the B_{12}-MS sequence GLNC^{247}ALGP, the following BHMT sequence GVNC^{217}HFDP appeared to be similar. Thus, to determine whether Cys217, Cys299, and Cys300 were critical for BHMT activity and Zn binding, the plasmid pTrc99A-hBHMT was mutated to create the following amino acid changes: Cys217Ala, Cys217Ser, Cys217His, Cys299Ala, Cys299Ser, Cys300Ala, and Cys300Ser. The plasmids were transformed into *E. coli* strains JM105 and J5-3. Transformed J5-3 cells were plated on defined media that contained 10 mM dimethylacetothetin and 3 mM IPTG, but which lacked methionine. After 2 days, the plates were examined for growth and compared to a sample of J5-3 transformed with wild-type pTrc99A-hBHMT. All seven mutants failed to support growth, suggesting that the BHMT enzyme was either inactive or perhaps improperly folded and rapidly degraded (Figure 2B). To eliminate the latter possibility and verify that the former was true, Western analysis was performed on the crude lysates of IPTG-induced JM105 transformants bearing either

wild-type or mutant pTrc99A-hBHMT constructs. The results showed that full-length mutant proteins were abundantly expressed (not shown); however, the lysates contained no BHMT activity as measured by the betaine-dependent in vitro assay (Table 2).

After developing the wild-type BHMT-intein/chitin-binding fusion construct (pTYB4-hBHMT) and successfully purifying the wild-type enzyme, pTYB4-hBHMT was mutated to create the following amino acid changes: Cys217Ala, Cys217Ser, Cys217His, Cys299Ala, Cys299Ser, Cys300Ala. The plasmids were transformed into *E. coli* strain BL21(DE3) and the mutant BHMT proteins overexpressed and purified using the IMPACT T7 system. ICP analysis for Zn indicated that the ability of the mutant proteins to bind Zn was greatly diminished (Table 2). The Cys to Ala mutants at Cys217, Cys299, and Cys300 contained 0.05, 0.22, and 0.08 Zn per mole of protein monomer, respectively. The Cys to Ser mutants at Cys217 and Cys299 contained 0.21 and 0.18 Zn per mole of protein monomer, respectively. All of the mutant proteins in this series were inactive as measured by the betaine-dependent radioassay. These results, and those obtained with the in vivo assay, were consistent with Cys217, Cys299, and Cys300 being Zn ligands for BHMT. As in the case of BHMT, when any of these conserved Cys residues were mutated to Ala in *E. coli* B_{12}-MS, the protein lacked methyltransferase activity and retained only 0.01, 0.02, and 0.15 Zn per mole of protein, respectively (*6*, *8*). In addition, extended X-ray fine structure analysis of *E. coli* B_{12}-MS showed that Zn was bound by three thiolate ligands and a fourth ligand that was either a nitrogen or an oxygen (*8*), confirming that the Zn in that protein was ligated by three thiolates (*8*). Although tenuous in the absence of crystal structure, it can be concluded that these conserved Cys residues are required for Zn binding in both BHMT and B_{12}-MS.

Based on the prior work outlined above on *E. coli* B_{12}-MS, we expected the Cys to Ala and Ser mutations at positions 217, 299, and 300 to diminish the Zn binding capability of BHMT; however, the ICP result for the Cys217His mutant was unexpected. Although Cys217His bound Zn as anticipated (0.66 Zn per mole of protein monomer), it was completely inactive by both the in vivo microbiological assay and the betaine-dependent in vitro radioassay. We also tried to measure the methyltransferase activity of Cys217His using the dimethylacetothetin-dependent radioassay. In addition to Cys217His, we also tried to measure the activity of Cys217Ser and Cys299Ser mutants using dimethylacetothetin because they too contained some Zn. We had previously determined that the reincorporation of Zn into the wild-type apoenzyme proceeded instantaneously with near-complete restoration of activity when nanomolar amounts of enzyme were incubated with 500 μM Zn chloride.[2] Therefore, the thetin-dependent in vitro radioassays were conducted in the presence of excess of Zn with the intention of providing enough Zn to occupy all of the active sites competent to bind the metal. Mutant proteins Cys217Ser and Cys299Ser exhibited 1/333 and 1/476 the activity of the wild-type enzyme, respectively, and Cys217His was completely inactive. These results suggest that the charge state of the Zn−ligand complex may be important for catalytic activity. The mechanism of methyl transfer for B_{12}-MS has been proposed to proceed through the formation of a zinc tetrathiolate complex by the attack of homocysteine on the Zn to displace the fourth nonthiolate ligand and give a complex with a net charge of −2 (*8*). Attack of homocysteine upon a Zn where one of the Cys ligands has been replaced with Ser or His could result in the formation of a trithiolate 4-coordinate Zn complex with a net charge of −1. A decreased net charge would predictably decrease the dissociate character of the complex and thus diminish the reactivity of homocysteine (*13*). Alternatively, the loss of activity could be the result of changes in active site structure upon substitution of the Zn ligands.

To determine if there were other enzymes that contained these three conserved Cys residues, GenBank was searched by a Pattern Hit Initiated Blast (*11*) using the amino acid sequence of human BHMT as the subject sequence and GGCC as the pattern. From this search, five sequences besides BHMT and *E. coli* B_{12}-MS were chosen and aligned by hand. The alignment revealed four conserved regions (A, B, C, and D) shared by all seven enzymes, and a fifth (C2) that was shared with all of the enzymes except BHMT (Figure 3). Regions A and B show little amino acid identity but significant similarity. For example, region A contains the distinct motif of four small hydrophobic residues, which is then followed by a DGG sequence in human BHMT, *E. coli* B_{12}-MS, and the two yeast open reading frames, whereas the other proteins have conservative substitutions at these positions. Region B, although also very limited in amino acid identity, displays a significant number of conservative substitutions as well. Neither region A nor region B has been studied and would make a good target for further investigation. Region C2 contains the motif YPNSG, which is found in all of the sequences except BHMT. This sequence also has been recently identified in a number of other plant and bacterial enzymes (*14*). Regions C and D are of particular interest because they contain the putative Zn ligands of BHMT and B_{12}-MS. Analysis of the alignment in regions C and D reveals the following motif which we propose to be the signature of a family of Zn-dependent thiol/selenol-dependent methyltransferases: G[ILV]NCX(20,100)[ALV]X-(2)[ILV]GGCCX(3)PX(2)I. This motif suggests that in both regions the Zn ligands are proceeded by a hydrophobic region, and the conserved Pro in region D suggests that this Zn-binding region may be positioned on a loop.

In the above-described motif, the Cys residue in region C is proceeded by a strictly conserved Asn residue. With the intent of determining the importance of this conserved Asn residue in the Zn-binding region, Asn216 was mutated to Ala and Cys. The Ala mutant was created to determine if Asn216 was essential for activity, and the Cys mutant was created to determine if it would result in a catalytically inactive enzyme that would bind Zn using Asn216Cys as the fourth thiolate ligand and thus prevent homocysteine from binding to the enzyme. However, both enzymes were active by both the in vivo and in vitro activity assays. These results indicate that while Asn216 is conserved, it is not essential for activity. Having examined Asn216 and elucidated the function of the Cys residues in regions C and D, we are currently investigating the role other conserved residues in

[2] Millian, N. S., and Garrow, T. A., unpublished data.

these regions have on the structure, function, and substrate specificity of BHMT.

In addition to finding residues involved in Zn binding, we wanted to identify other residues required for substrate binding and catalysis. Wild-type BHMT exhibits a parabolic pH dependence (pH 7.5 optima), indicating the existence of a general acid and base or two ionizable residues important for activity (*16*). Although the BHMT reaction does not result in a net change of proton stoichiometry, the conversion of homocysteine to methionine and of betaine to dimethylglycine results in the loss and gain of a proton, respectively. It is possible that one or more amino acid residues on BHMT facilitate this process and that mutation of these putative residues to Ala would be expected to result in a dramatic loss of activity. Toward this end, three polar amino acids near the Cys ligands of Zn (Asp220, Glu303, and His306) were mutated to Ala and the mutant proteins analyzed by the in vivo microbiological assay. However, all three mutants supported growth as well as the wild-type enzyme, indicating that none of these three residues were functioning as a general acid or base or were critical for activity.

In addition to a possible general acid−base function, we wanted to determine if the His306Ala mutation had any effect on Zn binding to BHMT since it is proximal to Cys299 and Cys300 and could function as a putative fourth ligand until displaced by the incoming thiol substrate. Thus, the His306Ala mutant was overexpressed and purified. ICP analysis revealed that His306Ala contained 0.76 Zn per mole of protein monomer, and had a specific activity that was about half that of the wild-type enzyme. H306 could be the labile fourth ligand of Zn or part of a secondary scaffolding that indirectly aids Zn binding. Spectrometric and mutagenic studies are currently underway to determine the function of His306.

ACKNOWLEDGMENT

We thank Anthony Spatz for his technical assistance, and Chris Perry for his help in preparing the manuscript.

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BI991003V